APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1992, p. 3834–3836
0099-2240/92/123834-03$02.00/0

Vol. 58, No. 12

Electrochemical Ag^+ for Preservative Use

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Received 15 June 1992/Accepted 29 September 1992

In contact experiments with different experimental conditions, electrochemical Ag^+ solutions exhibited better antimicrobial effectiveness against bacteria, a yeast species, and a mold than did analogous silver solutions from inorganic salts. The particular characteristics of electrochemical Ag^+, such as the mode of action, effectiveness at low concentrations, and stability, indicate that Ag^+ could be used effectively in preservatives.

The antimicrobial activity (oligodynamic action) of small quantities of metals (13), recognized since the nineteenth century, has been the basis for the development of many antimicrobial processes and products (8). Among the metals, silver and silver salts have been widely employed, particularly for water disinfection (11). More recently, silver has been utilized for topical applications, and a 1 to 3% silver sulfadiazine cream is used worldwide to prevent infection of burn wounds and to treat postinfection skin conditions (7). The Ag ion has been investigated in terms of its activity against different microorganisms, optimum Ag^+ concentration, time, pH, and temperature (11, 14), and cellular targets (9). With regard to the mechanism of action, Goetz (5) stated that silver is microbicidal only if it is in the ionic state, and Rochart and Uzdins (10) pointed out the selective absorption of these ions by the cell surface. Silver compounds that ionize poorly do not provide enough ions are still good antiseptics (6). Silver may be used as a metal, but the active agent appears to be the ions produced (7). Although different salts appear to work by the same mechanism, i.e., by supplying the silver ion at different rates, there is a difference of opinion as to the effectiveness of the silver in relation to microbial attack (15) and microbial alteration (4). Reviewing the bactericidal effectiveness of silver, Woodward (14) ascertained several discrepancies in the literature. For instance, some authors have reported that the silver activity is affected by chlorides, sulfides, and phosphates, whereas others have found no difference between silver nitrate and electrolytically produced silver (14). Using the broth dilution susceptibility test with 16 gram-positive and -negative microorganisms, Berger et al. (1, 2) found the bacteriostatic and bactericidal concentrations of electrochemical Ag^+ to be 10 to 100 times lower than those of silver sulfadiazine. They similarly showed that the activity of anode-derived Ag^+ against yeast in culture experiments was higher than that of silver compounds. It is not yet completely understood whether the microbiological effectiveness of silver solutions can be correlated to the source of silver, particularly in contact experiments. Since we are interested in the potential use of Ag in preservatives, for instance, in pharmaceutical or cosmetic preparations, the aim of our study was to investigate the microbicidal activity of silver by comparing the effectiveness of an electrochemically obtained solution with that from inorganic salts against bacteria, a yeast species, and a mold in contact experiments.

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MATERIALS AND METHODS

Sterile, purified (distilled on permanganate) water was used for all solutions and microbial assays. To prepare the $AgNO_3$ solutions, we used analytical-grade silver nitrate that had been heated at 120°C for 2 h. In some experiments, we used a silver chloride solution obtained from a silver nitrate solution with hydrochloric acid. The precipitate was washed until the NO_3^- disappeared and then poured into water to form a saturated solution. The supernatant silver chloride solution was filtered off, and the Ag^+ concentration was determined. The silver solution obtained by the electrochemical method [indicated as Ag(e)] was prepared by using a 4.5-V battery connected with two silver electrodes (length, 8 cm; area of cross section, 4 mm^2; distance between electrodes, 3 cm) in a 100-ml glass cylinder. This device supplied an Ag(e) solution of $\approx 2 \times 10^{-5}$ M after 15 min. This solution was tested with I^- and starch-water to ensure that no hydrogen peroxide was present. The Ag^+ concentration was determined at room temperature by using an Ag_2S electrode, and all potential readings were recorded after stabilization to ±0.1 mV/min. The function of the electrode was examined by measuring the potentials (in millivolts) of 10^{-3} to 10^{-7} M $AgNO_3$ solutions ($593.92 + 61.37 \times \log C$, where C is the molar concentration; $n = 10$; $R = 1.00$). All silver solutions were protected from exposure to light. All

TABLE 1. Microbicidal activity of Ag(e) and $AgNO_3$ solutions against *E. coli*, *P. aeruginosa*, *C. albicans*, and *A. niger*[a]

Species	Agent (M)	Intercept	K	R^2	D (min)	E
E. coli	Ag(e) (10^{-5})	5.346	−0.242	0.950	4.1	1.17
	$AgNO_3$ (10^{-5})	5.421	−0.205	0.913	4.9	
P. aeruginosa	Ag(e) (10^{-5})	4.548	−0.026	0.987	38.7	1.61
	$AgNO_3$ (10^{-5})	4.429	−0.016	0.875	62.5	
C. albicans	Ag(e) (10^{-6})	4.794	−0.016	0.956	62.5	∞
	$AgNO_3$ (10^{-6})					
A. niger	Ag(e) (10^{-6})	5.359	−0.021	0.979	47.6	∞
	$AgNO_3$ (10^{-6})					

[a] Experiments were conducted at 25°C and pH 6.5. Microbial suspensions contained 10^6 cells per ml. Intercept, intercept of regression curve; K, slope of the regression curve; R^2, correlation coefficient from regression; D, time required to achieve 90% reduction in viable cells; E, $K_{Ag(e)}/K_{AgNO_3}$. $AgNO_3$ showed no activity against *C. albicans* and *A. niger*.

TABLE 2. Ag(e) and $AgNO_3$ dilution exponents with different species[a]

Species	η of:	
	Ag(e)	$AgNO_3$
E. coli	0.12	0.19
P. aeruginosa	0.24	0.66
C. albicans	0.077	∞

[a] Experiments were conducted at 25°C and pH 6.5. η values were computed with Ag(e) and $AgNO_3$ concentrations of 10^{-5} and 10^{-6} M.

pH values were adjusted with 0.01 M citric acid-citrate buffer.

Strains. The tests were carried out against gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*), a yeast (*Candida albicans*), and a mold (*Aspergillus niger*); the strains were isolated from various clinical specimens and identified by standard methods. For the inoculum preparation and for viable cell counting, expressed as CFU, *E. coli* and *P. aeruginosa* were grown in BHI agar (BBL) for 24 h at 37°C and *C. albicans* and *A. niger* were grown in Sabouraud dextrose agar (BBL) for 48 h at 37°C. The cells were washed before use. The cell suspension concentration was calculated by measuring the optical density at 540 nm.

Survivor-time curves. The microbicidal activity was investigated with direct contact at 25°C. The contact times, five per experiment, were selected in relation to strain sensitivity. After contact of 9 ml of a suitable silver solution with 1 ml of a suitable microbial suspension (in H_2O at pH 7) the survivors were counted. A 0.1-ml sample was diluted 1:100, and then 1 ml or 0.1 ml was dispersed into 10 ml of agar medium. Care was taken to dilute the samples appropriately so that silver ion activity was immediately arrested. To avoid interference, no inactivators were used. All experiments were repeated three times.

Statistical analysis. To test for statistically significant differences among the activities of silver solutions, the analysis of variance was determined. A P value of <0.05 was considered to be statistically significant.

TABLE 3. Influence of ions on the antimicrobial activities of Ag^+ solutions[a]

Agent (M)	*E. coli*		*C. albicans*	
	% Survivors[b]	P[c]	% Survivors[b]	P[c]
Ag(e) (10^{-6})	0.01 ± 0.005		2 ± 1.0	
AgCl (10^{-6})	0.02 ± 0.012	0.17	6 ± 1.68	0.0014
$AgNO_3$ (10^{-6})	0.03 ± 0.014	0.023	7.4 ± 2.07	0.0008
Ag(e) (10^{-6}) + KNO_3 (10^{-6})	0.05 ± 0.02	0.004	10 ± 1.58	0.0000
Ag(e) (10^{-7})	0.2 ± 0.079		30 ± 10	
AgCl (10^{-7})	0.4 ± 0.15	0.035	35 ± 11.72	0.48
$AgNO_3$ (10^{-7})	0.5 ± 0.14	0.033	40 ± 16.2	0.27
Ag(e) (10^{-7}) + KNO_3 (10^{-7})	0.8 ± 0.24	0.0008	41 ± 11.4	0.14

[a] Experiments were conducted at 25°C and pH 6.5. Suspensions of *E. coli* contained 10^6 cells per ml, and the contact time was 1 h. Suspensions of *C. albicans* contained 10^4 cells per ml, and the contact time was 3 h.

[b] Data are means ± standard deviations.

[c] P value refers to the activity of silver from each different source compared with Ag(e) activity.

TABLE 4. Effect of temperature on the microbicidal activities of Ag(e) and $AgNO_3$ against *C. albicans*[a]

Temp (°C)	Mean % survivors ± SD in cultures treated with:	
	Ag(e)	$AgNO_3$
35	1.4 ± 0.55	105.2 ± 2.59
25	9.2 ± 4.87[b]	105.6 ± 2.30

[a] Experiments were conducted at the indicated temperature and pH 6.5. Contact time was 30 min. Ag(e) and $AgNO_3$ were both used at 10^{-6} M. The initial culture contained 10^6 *C. albicans* cells per ml.

[b] $P = 0.0074$; comparison of the different Ag(e) activities at 25 and 35°C.

RESULTS

The silver solutions showed very different activities against different microbial species. After 60 min of contact at pH 6.5 with suspensions (10^6 cells per ml) of *E. coli*, *P. aeruginosa*, *C. albicans*, and *A. niger*, a 10^{-6} M Ag(e) solution resulted in survivor percentages of 0 to 1%, 14 to 16%, 8.5 to 15%, and 6.4 to 10%, respectively. The activities of Ag(e) and $AgNO_3$ solutions against different microbial species are compared in Tables 1 and 2. The calculated slope of the regression curve, time required to achieve a 90% reduction in viable cells, and $K_{Ag(e)}/K_{AgNO_3}$ show that, although *E. coli*, *P. aeruginosa*, *C. albicans*, and *A. niger* displayed different sensitivities, the activity of the Ag(e) solution was always higher; moreover, the η values {[(log death time at concentration C_2) − (log death time at concentration C_1)] × $1/(\log C_1/C_2)$} demonstrated that Ag(e) was less influenced by dilution than was $AgNO_3$ (Table 2). It has been shown that the biological activity of electrochemical Ag^+ is similar to that of Ag^+ from inorganic salts (12); nevertheless, quantitative data from contact experiments (Table 3), comparing Ag(e) with $AgNO_3$, AgCl, and Ag(e)-KNO_3 solutions against *E. coli* and *C. albicans* showed that the microbiological effectiveness of Ag^+ was really influenced by NO_3 and Cl^-. The influence of these ions was well displayed by the variation of the activities of 10^{-6} M Ag^+ from different sources against *C. albicans*. The data reported in Table 4 show that the Ag(e) activity improved with rising temperature. The microbicidal effectiveness of Ag(e) appeared more affected by pH and was enhanced in alkaline medium; the activity-pH correlation and degree of activity were more evident with Ag(e) than with analogous $AgNO_3$ solutions (Table 5). Ag(e) action was affected by microbial concentration, but Ag(e) was significantly more effective than $AgNO_3$ (Table 6). The percentages of survivors (± standard deviations) in cultures treated with a freshly prepared Ag(e) solution and another solution prepared 30 days

TABLE 5. Effect of pH on the microbicidal activities of Ag(e) and $AgNO_3$ against *C. albicans*[a]

pH	Mean % survivors ± SD in cultures treated with:	
	Ag(e)	$AgNO_3$
5	18.4 ± 2.32	105.8 ± 17.34
6.5	9.2 ± 4.87	105.6 ± 2.30
7.5	0.4 ± 0.55[b]	44.6 ± 4.45

[a] Experiments were conducted at 25°C. Contact time was 30 min. Ag(e) and $AgNO_3$ were both used at 10^{-6} M. The initial culture contained 10^6 *C. albicans* cells per ml.

[b] $P = 0.0000$; comparison of the Ag(e) activities with different pH values.

TABLE 6. Effect of microbial concentration on the microbicidal activities of Ag(e) and $AgNO_3$ against *C. albicans*[a]

Inoculum (cells/ml)	Mean % survivors ± SD in cultures treated with:	
	Ag(e)	$AgNO_3$
10^6	9.2 ± 4.87	105.6 ± 2.30
10^7	18 ± 4.18[b]	104.6 ± 3.51

[a] Experiments were conducted at 25°C and pH 6.5. Contact time was 30 min. Both Ag(e) and $AgNO_3$ were used at 10^{-6} M.

[b] $P = 0.015$; comparison of the Ag(e) activities with different concentrations of organisms.

before testing were 0.4% ± 0.55% and 0.2% ± 0.48%, respectively ($P = 0.544$).

DISCUSSION

Our experiments showed that the contact antimicrobial activity of Ag(e) was superior to that of $AgNO_3$ against gram-positive and -negative bacteria, *C. albicans*, and a filamentous mycete. Our contact tests confirmed the excellent antibacterial spectrum and the high potency of electrically generated silver demonstrated previously in broth dilution susceptibility tests (1). The Woodward report (14) contained data regarding the activity of silver against only *E. coli*; because *E. coli* is highly sensitive to silver ions, it could be inadequate for differential analysis, as was observed in our experiments. Indeed, we preferred to use the less sensitive species *C. albicans* to show that the microbial effectiveness of silver is reduced by the NO_3^- ion. Exactly how ions influence microbiological activity is not known. Chlorides may act by forming complexes, and the low solubility of silver sulfides may have some effect, but these explanations would not apply to the effects of phosphates and nitrates or to pH dependence. In conclusion, even though Ag(e) and Ag^+ from inorganic salts have similar, apparently membrane-related activities (12), the microbicidal activity of silver is significantly ion influenced. The main problem in pharmaceutical and particularly in cosmetic preparations is to develop a safe and stable preservative that is also very active against a broad spectrum of microorganisms. Anodic silver ions are very effective agents at low concentrations without any detrimental effect upon normal mammalian cells (2), and the concentrations needed to inhibit the bacteria in in vitro experiments have been confirmed by clinical data (7). Our experimental results confirm the potential of Ag(e) for use as a preservative; this anion-free preservative system might show a reduced interference with the other materials used in most formulations.

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